

**This Page Is Inserted by IFW Operations  
and is not a part of the Official Record**

## **BEST AVAILABLE IMAGES**

**Defective images within this document are accurate representations of the original documents submitted by the applicant.**

**Defects in the images may include (but are not limited to):**

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 9/20, 9/28, 15/55, 15/56 // C11D 3/386</b>	<b>A1</b>	<b>(11) International Publication Number: WO 94/14951</b> <b>(43) International Publication Date: 7 July 1994 (07.07.94)</b>
<b>(21) International Application Number:</b> PCT/DK93/00441 <b>(22) International Filing Date:</b> 22 December 1993 (22.12.93)  <b>(30) Priority Data:</b> 1542/92 23 December 1992 (23.12.92) DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> NIELSEN, Egon [DK/DK]; Niels W. Gades Gade 33, st., DK-2100 Copenhagen Ø (DK). RASMUSSEN, Grethe [DK/DK]; Theklavej 15, st. tv., DK-2400 Copenhagen NV (DK). HALKIER, Torben [DK/DK]; Vodroffsvej 14A.7, DK-1900 Frederiksberg C. (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).		<b>(81) Designated States:</b> BR, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title: RECOMBINANT LIPASE AND ALPHA-AMYLASE VARIANTS</b>  <b>(57) Abstract</b>  The present invention relates to lipase and $\alpha$ -amylase variants, stabilized towards the inactivation caused by peroxidase systems, in which variants a naturally occurring tyrosine residue has been deleted or substituted with a different amino acid residue at one or more positions. The invention also relates to a method of stabilizing a lipase or an $\alpha$ -amylase towards the inactivation caused by peroxidase systems, and detergent compositions comprising a lipase and/or an $\alpha$ -amylase variant of the invention.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

RECOMBINANT LIPASE AND ALPHA-AMYLASE VARIANTS

## TECHNICAL FIELD

The present invention relates to lipase and  $\alpha$ -amylase variants, stabilized towards the inactivation caused by peroxidase systems, in which lipase and  $\alpha$ -amylase variants a naturally occurring tyrosine residue has been deleted or substituted with a different amino acid residue at one or more positions.

The invention also relates to a method of stabilizing a lipase or an  $\alpha$ -amylase towards the inactivation caused by peroxidase systems, and detergent compositions comprising a lipase and/or an  $\alpha$ -amylase variant of the invention.

## BACKGROUND ART

Peroxidases (E.C. 1.11.1.7) are enzymes that catalyse the oxidation of a substrate (an electron or hydrogen donor) with hydrogen peroxide. Such enzymes are known from microbial, plant and animal origins, e.g. peroxidase from Coprinus cinereus (cf. e.g. EP Patent Application 179,486). They are typically hemoproteins, i.e. they contain a heme as a prosthetic group.

Use of peroxidase together with hydrogen peroxide or a hydrogen peroxide precursor has been suggested e.g. in bleaching of pulp for paper production, in treatment of waste water from pulp production, for improved bleaching in laundry detergents, for dye transfer inhibition during laundering, and for lignin modification, e.g. in particle board production.

Peroxidase systems (also designated POD systems) comprising a peroxidase or a compound exhibiting peroxidase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent, are used for preventing coloured substances, which leach from dyed fabrics, to deposit on other fabrics present in the same wash (this phenomenon is commonly known as

dye transfer). Detergent compositions or wash liquors comprising such peroxidase systems have been described in e.g. International Patent Applications WO 92/18687 and WO 92/18683.

A major drawback in applying such peroxidase systems to detergent compositions is that the enzymes present in such compositions may be strongly affected by the peroxidase system, thereby hampering the washing performance of the detergent composition.

#### SUMMARY OF THE INVENTION

10 It has now surprisingly been found that lipases and  $\alpha$ -amylases may be stabilized towards inactivation caused by peroxidase systems, by deletion or substitution of one or more naturally occurring tyrosine residues with a different amino acid residue.

15 Accordingly, the invention provides a lipase and/or an  $\alpha$ -amylase variant, in which one or more naturally occurring tyrosine residues have been deleted or substituted with a different amino acid residue.

In another aspect, the invention provides a method  
20 of stabilization of a lipase and/or an  $\alpha$ -amylase variant towards inactivation caused by a peroxidase system, in which method one or more naturally occurring tyrosine residues are deleted or substituted with a different amino acid residue.

In a further aspect, the invention provides detergent  
25 gent compositions comprising a lipase and/or an  $\alpha$ -amylase variant of the invention.

In a yet further aspect, the invention provides detergent additives comprising a lipase and/or an  $\alpha$ -amylase variant of the invention.

### DETAILED DISCLOSURE OF THE INVENTION

The present invention provides novel lipase and  $\alpha$ -amylase variants, stabilized towards inactivation caused by peroxidase systems.

5 In the context of this invention, a stabilized lipase or  $\alpha$ -amylase variant is a lipase or an  $\alpha$ -amylase having improved stability towards inactivation caused by peroxidase systems, when compared to the parent lipase or  $\alpha$ -amylase.

#### Amino Acids

10 As abbreviations for amino acids the following symbols are used:

	A	=	Ala	=	Alanine
	C	=	Cys	=	Cysteine
	D	=	Asp	=	Aspartic acid
15	E	=	Glu	=	Glutamic acid
	F	=	Phe	=	Phenylalanine
	G	=	Gly	=	Glycine
	H	=	His	=	Histidine
	I	=	Ile	=	Isoleucine
20	K	=	Lys	=	Lysine
	L	=	Leu	=	Leucine
	M	=	Met	=	Methionine
	N	=	Asn	=	Asparagine
	P	=	Pro	=	Proline
25	Q	=	Gln	=	Glutamine
	R	=	Arg	=	Arginine
	S	=	Ser	=	Serine
	T	=	Thr	=	Threonine
	V	=	Val	=	Valine
30	W	=	Trp	=	Tryptophan
	Y	=	Tyr	=	Tyrosine
	B	=	Asx	=	Asp (D) or Asn (N)
	Z	=	Glx	=	Glu (E) or Gln (Q)
	X	=	an arbitrary amino acid		
35	*	=	deletion or absent amino acid		

#### Peroxidase Activity

In the context of this invention, the enzymatic activity of peroxidases is expressed in "Peroxidase Units" (PODU). In the presence of hydrogen peroxide peroxidases (E.C.

1.11.1.7) catalyse the dehydrogenation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS). The greenish-blue colour produced is monitored photometrically at 418 nm. One PODU is the amount of enzyme which, under standard conditions (i.e. pH 7.0; hydrogen peroxide as substrate; 0.1 M phosphate buffer; an incubation temp. of 30°C; an incubation time of 3 min. measured kinetically) catalyses the conversion of 1  $\mu$ mol of hydrogen peroxide per minute.

#### Lipase Activity

10 In the context of this invention, the enzymatic activity of lipases is expressed in Lipase Units. A Lipase Unit (LU) is the amount of enzyme which under standard conditions, i.e. 30.0°C; pH 7.0; tributyrine substrate, liberates 1  $\mu$ mol titratable butyric acid per minute.

#### 15 $\alpha$ -amylase Activity

The  $\alpha$ -amylase activity is measured as absorption/ml at 620 nm using Phadebas tablets (Phadebasv® Amylase Test; Pharmacia Diagnostics, SW). The assay is carried out at 60°C.

#### Peroxidase Systems

20 In the context of this invention, a peroxidase system is a system comprising a peroxidase or a compound exhibiting peroxidase activity, a source of hydrogen peroxide, and a peroxidase enhancing agent. Such peroxidase systems have been used for obtaining a dye transfer inhibition and have been  
25 described in e.g. International Patent Applications WO 92/18687 and WO 92/18683.

In such a peroxidase system, the peroxidase or the compound exhibiting peroxidase activity may be any peroxidase comprised by the enzyme classification EC 1.11.1.7, or any  
30 fragment derived therefrom, exhibiting peroxidase activity, or synthetic or semisynthetic derivatives thereof (e.g. porphyrin ring systems or microperoxidases, cf. e.g. US Patent 4,077,768, EP Patent Application 537,381, International Patent Ap-

plications WO 91/05858 and WO 92/16634). Such peroxidases are known from microbial, plant and animal origins.

The peroxidase may be producible by plants (e.g. horseradish or soy bean peroxidase) or microorganisms such as  
5 fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g. Fusarium, Humicola, Tricoderma, Myrothecium, Verticillium, Arthromyces, Caldariomyces, Ulocladium, Embellisia, Cladosporium or Dreschlera, in particular Fusarium  
10 oxysporum (DSM 2672), Humicola insolens, Trichoderma resii, Myrothecium verrucana (IFO 6113), Verticillium alboatrum, Verticillium dahliae, Arthromyces ramosus (FERM P-7754), Caldariomyces fumago, Ulocladium chartarum, Embellisia allior Dreschlera halodes.

15 Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. Coprinus, Phanerochaete, Coriolus or Trametes, in particular Coprinus cinereus f. microsporus (IFO 8371), Coprinus macrorhizus, Phanerochaete chrysosporium (e.g. NA-12) or Trametes  
20 (previously called Polyporus), e.g. T. versicolor (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. Rhizopus or Mucor, in particular Mucor hiemalis.

25 Some preferred bacteria include strains of the order Actinomycetales, e.g. Streptomyces spheroides (ATCC 23965), Streptomyces thermoviolaceus (IFO 12382) or Streptoverticillium verticillium ssp. verticillium

Other preferred bacteria include Bacillus pumilus  
30 (ATCC 12905), Bacillus stearothermophilus, Rhodobacter sphaeroides, Rhodomonas palustri, Streptococcus lactis, Pseudomonas purrocinia (ATCC 15958) or Pseudomonas fluorescens (NRRL B-11).

Further preferred bacteria include strains belonging to Myxococcus, e.g. M. virescens.

35 Other potential sources of useful particular peroxidases are listed in Saunders B C, op. cit., pp. 41-43.



The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a Coprinus sp., in particular C. macrorrhizus or C. cinereus according to WO 92/16634.

In the context of this invention, compounds exhibiting peroxidase activity comprise peroxidase active fragments derived from cytochromes, hemoglobin or peroxidase enzymes, and synthetic or semisynthetic derivatives thereof, e.g. iron porphins, iron porphyrins, and iron phthalocyanine and derivatives thereof.

In a peroxidase system, the enhancer may be an oxidizable substrate e.g. metal ions or phenolic compounds such as 7-hydroxycoumarin (7HCm), vanillin (VAN), and p-hydroxybenzenesulfonate (PHBS), described in e.g. International Patent Applications WO 92/18683 and WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 26 (7), pp. 1291-1301 (cf. Table 1 in particular), and Saunders B C, et al., Peroxidase, London, 1964, p. 141 ff. or 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), described in applicant's copending DK Patent Application No. 9201441.

### Lipases

In a preferred embodiment, the lipase of the invention is obtainable from a strain of Humicola, e.g. H. lanuginosa, H. brevispora, H. brevis var. thermoidea, or H. insolens. Lipases obtainable from Humicola are described in e.g. US Patent 4,810,414, EP Application 305,216 and International Patent Application WO 89/01969, which publications are hereby included by reference.

In another specific embodiment, the lipase is obtainable from a strain of Pseudomonas, e.g. Ps. cepacia; Ps. fragi, Ps. stutzeri, or Ps. fluorescens. Lipases obtainable from Pseudomonas are described in e.g. International Patent  
5 Publication 89/04361, which publication is hereby included by reference.

In a third specific embodiment, the lipase is obtainable from a strain of Fusarium, e.g. F. oxysporum. Lipases obtainable from Fusarium are described in e.g. EP  
10 Publication 130,064 and EP Publication 395,678, which publications are hereby included by reference.

In further specific embodiments, the lipase is obtainable from a strain of Rhizomucor, e.g. Rhizomucor miehei, or a strain of Candida, e.g. C. antarctica, or C. cylindracea  
15 (also called C. rugosa), or a strain of Chromobacterium, e.g. C. viscosum.

In a more preferred embodiment, a lipase variant of the invention is a Humicola lanuginosa lipase having an amino acid sequence as described in EP Publication 305,216 (in which  
20 publication the amino acid sequence is presented in Fig. 5), which sequence has been changed in one or more of the following positions: 16, 21, 53, 138, 164, 171, 194, 213, 220, 261.

#### Amylases

In a preferred embodiment, the  $\alpha$ -amylase variant of  
25 the invention is obtainable from a strain of Bacillus or a strain of Aspergillus.

In a more specific embodiment, the  $\alpha$ -amylase variant is obtainable from a strain of B. licheniformis. The amino acid sequence for the B. licheniformis 584  $\alpha$ -amylase (Stephens et al.)  
30 appears from J. Bacteriol. 1984 158 369-372, and J. Bacteriol. 166, 635-643, 1986, FR 2665178 or EP 410498. Thus, the tyrosine positions are: 10, 14, 31, 46, 56, 59, 62, 77, 98, 150, 158, 175, 193, 195, 198, 203, 219, 262, 273, 290, 302, 348, 358, 363, 367, 394, 396, 402, 439, 480.

In another specific embodiment, the  $\alpha$ -amylase variant is obtainable from a strain of B. amyloliquefaciens. The amino acid sequence for the B. amyloliquefaciens  $\alpha$ -amylase (Takkinen et al.) appears from J. Biol Chem. 1983 258 1007-1013.

5 In a third specific embodiment, the  $\alpha$ -amylase variant is obtainable from a strain of B. stearrowthermophilus. The amino acid sequence for the B. stearrowthermophilus  $\alpha$ -amylase appears from J. Bacteriol. 166, 635-643, 1986.

In a fourth specific embodiment, the  $\alpha$ -amylase  
10 variant is obtainable from a strain of A. niger. The amino acid sequence for the A. niger  $\alpha$ -amylase appears from DK Patent Application 5126/87.

In further specific embodiments,  $\alpha$ -amylase variants of the invention are chimeric  $\alpha$ -amylases. Chimeric  $\alpha$ -amylases  
15 are disclosed in e.g. EP Patent Publication 252,666.

#### Methods of Stabilizing Lipases and $\alpha$ -amylases

The present invention provides a method of stabilizing lipases and  $\alpha$ -amylases towards inactivation caused by peroxidase systems, by which method one or more naturally  
20 occurring tyrosine residues are deleted or substituted with a different amino acid residue.

#### Recombinantly Produced Lipases and $\alpha$ -amylases

In the past, numerous processes have been developed for the production of polypeptides or proteins by means of the  
25 recombinant DNA technology. Mostly used for this purpose are E. coli, Bacillus subtilis, Saccharomyces cerevisiae and different Aspergillus strains, e.g. A. oryzae and A. niger. Especially the Aspergillii are attractive candidates as host microorganisms for recombinant DNA vectors being well-characterized  
30 and widely used microorganisms for the commercial production of enzymes. In Aspergillus oryzae, methods have been developed for transformation of the organism, and production of several enzymes, among these the Humicola lanuginosa and Rhizomucor miehei lipases (vide e.g. European Patent Applications 238,023

and 305,216, and International Patent Application WO 89/01969), which publications are hereby included by reference.

#### Expression of Polypeptides Biosynthetically

Upon transformation of an organism where the intention is production of a polypeptide or a protein, a DNA sequence is introduced into the organism. The sequence contains the coding region of the gene of interest flanked by transcription/translation start signals and transcription/translation termination signals. The coding region contains units of three base pairs, called codons, which upon translation of the transcribed gene are translated into amino acids, which again are assembled to give the polypeptide of interest.

#### Introducing Mutations in Polypeptides

By changing one or more specific codons in the coding region and transforming the host microorganism with these new coding regions, new polypeptides can be produced which differ from the original polypeptide by one or more amino acids. Such alterations can be introduced by means of a technique generally known as "site-directed in vitro mutagenesis". A number of methods have been published. An early method is described by Zoller & Smith, DNA 1984 3 (6) 479-488, and involves use of the single-stranded M13 bacteriophage. A preferred method using PCR (polymerase chain reaction) is described by Nelson & Long, Analytical Biochemistry, 1989 180 147-151. It involves a 3-step generation of a PCR fragment containing the desired mutation by using a chemically synthesized DNA oligonucleotide as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid. A third mutagenesis method takes advantage of restriction sites in the DNA coding region. By digesting the DNA with restriction enzymes at sites flanking the mutagenesis target, synthesizing a new fragment synthetically containing

the desired mutation and cloning this new fragment between the restriction sites, a mutant coding region can be constructed.

All methods are generally applicable to investigations in the field called protein engineering which deals with the development of polypeptides with new or altered characteristics.

Transformation and expression may be accomplished by methods known in the art, e.g. as described in European Patent Application 305,216, which specification is hereby included by reference.

The microorganisms able to produce a stabilized lipase or  $\alpha$ -amylase of this invention can be cultivated by conventional fermentation methods in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art. Purification and recovery of the stabilized lipase or  $\alpha$ -amylase may also be conducted in accordance with methods known per se.

#### Nucleotide Sequences, Expression Vectors And Microorganisms

This invention also relates to DNA nucleotide sequences encoding a stabilized lipase or  $\alpha$ -amylase of the invention. The stabilized lipase or  $\alpha$ -amylase may be expressed and produced when DNA nucleotide sequence encoding the lipase or  $\alpha$ -amylase is inserted into a suitable vector in a suitable host organism. The host organism is not necessarily identical to the organism from which the parent gene originated. The construction of the mutated genes, vectors and mutant and transformed microorganisms may be carried out by any appropriate recombinant DNA technique, known in the art.

The invention also relates to expression vectors and host organisms containing a DNA nucleotide encoding a stabilized lipase or  $\alpha$ -amylase of this invention.

### Detergent Compositions

According to the invention, the lipase and the  $\alpha$ -amylase variant may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000, ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50 % of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid or soap. It may also contain 0-

40 % of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyl dimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, alkyl-(N-methyl)-glucoseamide or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as cutinase, protease, cellulase, peroxidase, or oxidase.

10 The detergent may contain 1-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, 15 soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinyl- 20 pyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which 25 may comprise a  $H_2O_2$  source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone 30 type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric 35 acid derivative as e.g. an aromatic borate ester, and the com-

position may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. 7-11.

Particular forms of detergent compositions within the scope of the invention include:

- 1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising
  - linear alkylbenzenesulfonate  
15 (calculated as acid) 7 - 12%
  - alcohol ethoxysulfate  
(e.g. C<sub>12-18</sub> alcohol, 1-2 EO) or  
alkyl sulfate (e.g. C<sub>16-18</sub>) 1 - 4%
  - alcohol ethoxylate  
20 (e.g. C<sub>14-15</sub> alcohol, 7 EO) 5 - 9%
  - sodium carbonate (as Na<sub>2</sub>CO<sub>3</sub>) 14 - 20%
  - soluble silicate (as Na<sub>2</sub>O, 2SiO<sub>2</sub>) 2 - 6%
  - zeolite (as NaAlSiO<sub>4</sub>) 15 - 22%
  - sodium sulfate (as Na<sub>2</sub>SO<sub>4</sub>) 0 - 6%
  - 25 - sodium citrate/citric acid 0 - 15%  
(as C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>/C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>)
  - sodium perborate (as NaBO<sub>3</sub>·H<sub>2</sub>O) 11 - 18%
  - TAED 2 - 6%
  - carboxymethylcellulose 0 - 2%
  - 30 - polymers (e.g. maleic/acrylic acid  
copolymer, PVP, PEG) 0 - 3%
  - enzymes 0 - 5%
  - minor ingredients (e.g. suds  
supressors, perfume, optical  
35 brightener, photobleach) 0 - 5%



2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
5 - alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO) or alkyl sulfate (e.g. C <sub>16-18</sub> )	1 - 3%
- alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
10 - sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15 - 21%
- soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1 - 4%
- zeolite (as NaAlSiO <sub>4</sub> )	24 - 34%
- sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4 - 10%
- sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15 %
15 - carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
- enzymes	0 - 5%
20 - minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

25 - linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
- alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	7 - 14%
- soap as fatty acid (e.g. C <sub>16-22</sub> )	1 - 3%
30 - sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	10 - 17%
- soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	3 - 9%
- zeolite (as NaAlSiO <sub>4</sub> )	23 - 33%
- sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0 - 4%

- sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	8 - 16%
- TAED	2 - 8%
- phosphonate (e.g. EDTMPA)	0 - 1%
- carboxymethylcellulose	0 - 2%
5 - polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

10 4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
- alcohol ethoxylate (e.g. $\text{C}_{12-15}$ alcohol, 7 EO)	10 - 25%
15 - sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	14 - 22%
- soluble silicate (as $\text{Na}_2\text{O} \cdot 2\text{SiO}_2$ )	1 - 5%
- zeolite (as $\text{NaAlSiO}_4$ )	25 - 35%
- sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	0 - 10%
20 - carboxymethylcellulose	0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
- enzymes	0 - 5%
- minor ingredients (e.g. suds suppressors, perfume)	0 - 5%
25	

5) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
- alcohol ethoxylate (e.g. $\text{C}_{12-15}$ alcohol, 7 EO or $\text{C}_{12-15}$ alcohol, 5 EO)	12 - 18%
30	

- soap as fatty acid (e.g. oleic acid)	3 - 13%
- alkenylsuccinic acid ( $C_{12-14}$ )	0 - 13%
- aminoethanol	8 - 18%
- citric acid	2 - 8%
5 - phosphonate	0 - 3%
- polymers (e.g. PVP, PEG)	0 - 3%
- borate (as $B_4O_7$ )	0 - 2%
- ethanol	0 - 3%
- propylene glycol	8 - 14%
10 - enzymes	0 - 5%
- minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO 20 or $C_{12-15}$ alcohol, 5 EO)	3 - 9%
- soap as fatty acid (e.g. oleic acid)	3 - 10%
- zeolite (as $NaAlSiO_4$ )	14 - 22%
- potassium citrate	9 - 18%
- borate (as $B_4O_7$ )	0 - 2%
25 - carboxymethylcellulose	0 - 2%
- polymers (e.g. PEG, PVP)	0 - 3%
- anchoring polymers as e.g. lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
30 - glycerol	0 - 5%
- enzymes	0 - 5%

- minor ingredients  
(e.g. dispersants, suds suppressors, perfume,  
optical brighteners) 0 - 5%
- 7) A detergent composition formulated as a granulate having a  
5 bulk density of at least 600 g/l comprising
- fatty alcohol sulfate 5 - 10%
  - ethoxylated fatty acid monoethanolamide 3 - 9%
  - soap as fatty acid 0 - 3%
  - sodium carbonate (as  $\text{Na}_2\text{CO}_3$ ) 5 - 10%
  - 10 - soluble silicate (as  $\text{Na}_2\text{O}, 2\text{SiO}_2$ ) 1 - 4%
  - zeolite (as  $\text{NaAlSiO}_4$ ) 20 - 40%
  - sodium sulfate (as  $\text{Na}_2\text{SO}_4$ ) 2 - 8%
  - sodium perborate (as  $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ ) 12 - 18%
  - TAED 2 - 7%
  - 15 - polymers (e.g. maleic/acrylic acid copolymer,  
PEG) 1 - 5%
  - enzymes 0 - 5%
  - minor ingredients (e.g. optical brightener,  
suds suppressors, perfume) 0 - 5%
- 20 8) A detergent composition formulated as a granulate comprising
- linear alkylbenzenesulfonate  
(calculated as acid) 8 - 14%
  - ethoxylated fatty acid monoethanolamide 5 - 11%
  - soap as fatty acid 0 - 3%
  - 25 - sodium carbonate (as  $\text{Na}_2\text{CO}_3$ ) 4 - 10%
  - soluble silicate (as  $\text{Na}_2\text{O}, 2\text{SiO}_2$ ) 1 - 4%
  - zeolite (as  $\text{NaAlSiO}_4$ ) 30 - 50%
  - sodium sulfate (as  $\text{Na}_2\text{SO}_4$ ) 3 - 11%
  - sodium citrate (as  $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ ) 5 - 12%
  - 30 - polymers (e.g. PVP,  
maleic/acrylic acid copolymer, PEG) 1 - 5%

- enzymes 0 - 5%
  - minor ingredients (e.g. suds suppressors, perfume) 0 - 5%
- 9) A detergent composition formulated as a granulate comprising
- 5 - linear alkylbenzenesulfonate (calculated as acid) 6 - 12%
  - nonionic surfactant, 1 - 4%
  - soap as fatty acid 2 - 6%
  - sodium carbonate (as  $\text{Na}_2\text{CO}_3$ ) 14 - 22%
  - 10 - zeolite (as  $\text{NaAlSiO}_4$ ) 18 - 32%
  - sodium sulfate (as  $\text{Na}_2\text{SO}_4$ ) 5 - 20%
  - sodium citrate (as  $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ ) 3 - 8%
  - sodium perborate (as  $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ ) 4 - 9%
  - bleach activator (e.g. NOBS or TAED) 1 - 5%
  - 15 - carboxymethylcellulose 0 - 2%
  - polymers (e.g. polycarboxylate or PEG) 1 - 5%
  - enzymes 0 - 5%
  - minor ingredients (e.g. optical brightener, perfume) 0 - 5%
- 20 10) An aqueous liquid detergent composition comprising
- linear alkylbenzenesulfonate (calculated as acid) 15 - 23%
  - alcohol ethoxysulfate (e.g.  $\text{C}_{12-15}$  alcohol, 2-3 EO) 8 - 15%
  - 25 - alcohol ethoxylate (e.g.  $\text{C}_{12-15}$  alcohol, 7 EO or  $\text{C}_{12-15}$  alcohol, 5 EO) 3 - 9%
  - soap as fatty acid (e.g. lauric acid) 0 - 3%
  - aminoethanol 1 - 5%
  - 30 - sodium citrate 5 - 10%

- hydrotrope (e.g. sodium toluenesulfonate)	2 - 6%
- borate (as $B_4O_7$ )	0 - 2%
- carboxymethylcellulose	0 - 1%
- ethanol	1 - 3%
5 - propylene glycol	2 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

10 - linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO or $C_{12-15}$ alcohol, 5 EO)	6 - 12%
15 - aminoethanol	2 - 6%
- citric acid	8 - 14%
- borate (as $B_4O_7$ )	1 - 3%
- polymer (e.g. maleic/acrylic acid copolymer, anchoring polymers as e.g. 20 lauryl methacrylate/acrylic acid copolymer and CMC)	0 - 3%
- glycerol	3 - 8%
- enzymes	0 - 5%
- minor ingredients (e.g. hydrotropes, 25 dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

30 - anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
- nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%

- sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	8 - 25%
- soluble silicates (as $\text{Na}_2\text{O}$ , $2\text{SiO}_2$ )	5 - 15%
- sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	0 - 5%
- zeolite (as $\text{NaAlSiO}_4$ )	15 - 28%
5 - sodium perborate (as $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ )	0 - 20%
- bleach activator (TAED or NOBS)	0 - 5%
- enzymes	0 - 5%
- minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

10 13) Detergent formulations as described in 1) - 12) where the content of linear alkylbenzenesulfonate - or a part of it - is substituted by alkyl sulfate ( $\text{C}_{12}\text{-C}_{18}$ ).

14) Detergent formulations as described in 1) - 13) which contain a stabilized or encapsulated peracid either as an  
15 additional component or as a substitute for already specified bleach systems.

15) Detergent compositions as described in 3), 7), 9) and 12) where the content of perborate is substituted by percarbonate.

16) Detergent composition formulated as a nonaqueous detergent  
20 liquid comprising a liquid nonionic surfactant as e.g. linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The lipase and  $\alpha$ -amylase variants of the invention  
25 may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipase and  $\alpha$ -amylase variants may be added in an amount corresponding to

0.001-100 mg of lipase or  $\alpha$ -amylase variant per liter of wash liquor.



## CLAIMS

1. A lipase or an  $\alpha$ -amylase variant, stabilized towards inactivation caused by a peroxidase system comprising a peroxidase or a compound exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent, characterized in, that one or more naturally occurring tyrosine residues has/have been deleted or substituted with a different amino acid residue.

2. A lipase or an  $\alpha$ -amylase variant according to Claim 1, in which one or more tyrosine residue(s) has/have been substituted with a phenylalanine residue, a leucine residue, an isoleucine residue, a valine residue, a glutamine residue, an asparagine residue, a serine residue, a threonine residue, a glutamic acid residue, or a histidine residue.

3. A lipase variant according to either of claims 1-2, the lipase variant being obtainable from a strain of Humicola, Pseudomonas, Fusarium, Rhizomucor, or Candida.

4. A lipase variant according to claim 3, the lipase variant being obtainable from a strain of H. lanuginosa, H. brevispora, H. brevis var. thermoidea, H. insolens, Ps. cepacia, Ps. fragi, Ps. stutzeri, Ps. fluorescens, F. oxysporum, Rhizomucor miehei, C. antarctica, or C. cylindracea.

5. A lipase variant according to claim 4, the lipase variant being obtainable from a strain of H. lanuginosa.

6. A lipase variant according to claim 5, in which a naturally occurring tyrosine residue has been deleted or substituted in one or more of the following tyrosine positions: 16, 21, 53, 138, 164, 171, 194, 213, 220, 261.

7. An  $\alpha$ -amylase variant according to either of claims 1-2, the  $\alpha$ -amylase variant being obtainable from a strain of Bacillus or Aspergillus.

8. An  $\alpha$ -amylase variant according to claim 7, the  $\alpha$ -amylase variant being obtainable from a strain of B. licheniformis

9. An  $\alpha$ -amylase variant according to claim 8, in which a naturally occurring tyrosine residue has been deleted or substituted in one or more of the following positions: 10, 14, 31, 46, 56, 59, 62, 77, 98, 150, 158, 175, 193, 195, 198, 203, 219, 262, 273, 290, 302, 348, 358, 363, 367, 394, 396, 402, 439, 480.

10. A method of stabilization of a lipase or an  $\alpha$ -amylase towards inactivation caused by a peroxidase system comprising a peroxidase or a compound exhibiting peroxidase activity, a source of hydrogen peroxide and a peroxidase enhancing agent, characterized in, that one or more naturally occurring tyrosine residues is/are deleted or substituted with a different amino acid residue.

11. The method according to claim 10, characterized by substitution of one or more naturally occurring tyrosine residues with a phenylalanine residue, a leucine residue, an isoleucine residue, a valine residue, a glutamine residue, an asparagine residue, a serine residue, a threonine residue, a glutamic acid residue, or a histidine residue.

12. The method according to either of claims 10-11, the lipase being obtainable from a strain of Humicola, Pseudomonas, Fusarium, Rhizomucor, or Candida.

13. The method according to claim 12, the lipase being obtainable from a strain of H. lanuginosa, H. brevispora,

H. brevis var. thermoidea, H. insolens, Ps. cepacia, Ps. fragi,  
Ps. stutzeri, Ps. fluorescens, F. oxysporum, Rhizomucor miehei,  
C. antarctica, or C. cylindracea.

14. The method according to claim 13, the lipase  
5 being obtainable from a strain of Humicola lanuginosa.

15. The method according to claim 14, in which a  
naturally occurring tyrosine residue has been deleted or  
substituted in one or more of the following tyrosine positions:  
16, 21, 53, 138, 164, 171, 194, 213, 220, 261.

10 16. The method according to either of claims 10-11,  
the  $\alpha$ -amylase being obtainable from a strain of Bacillus or  
Aspergillus.

17. The method according to claim 16, the  $\alpha$ -amylase  
being obtainable from a strain of B. licheniformis.

15 18. The method according to claim 17, in which a  
naturally occurring tyrosine residue has been changed in one or  
more of the following positions: 10, 14, 31, 46, 56, 59, 62,  
77, 98, 150, 158, 175, 193, 195, 198, 203, 219, 262, 273, 290,  
302, 348, 358, 363, 367, 394, 396, 402, 439, 480.

20 19. A detergent composition comprising a lipase  
and/or an  $\alpha$ -amylase variant according to any of claims 1-9.

20. A detergent composition according to claim 19,  
which further comprises one or more other enzymes, in particu-  
lar proteases, cellulases, oxidases and/or peroxidases,  
25 conventionally used in detergents.

21. A detergent additive comprising a lipase and/or  
an  $\alpha$ -amylase variant according to any of claims 1-9, provided

in the form of a non-dusting granulate, a stabilized liquid, a slurry, or a protected enzyme.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 93/00441

## A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/20, C12N 9/28, C12N 15/55, C12N 15/56 // C11D 3/386  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, BIOSIS, CA, EPODOC

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 9311254 (NOVO NORDISK A/S), 10 June 1993 (10.06.93), page 5, line 3 - line 25 --	1-21
A	EP, A1, 0407225 (UNILEVER PLC), 9 January 1991 (09.01.91) --	1-21
A	WO, A2, 9100353 (GIST-BROCADES N.V.), 10 January 1991 (10.01.91) -- -----	1-21

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

## \* Special categories of cited documents

- "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

30 March 1994

31-03-1994

Name and mailing address of the ISA/  
 Swedish Patent Office  
 Box 5055, S-102 42 STOCKHOLM  
 Facsimile No. +46 8 666 02 86

Authorized officer

Carl Olof Gustafsson  
 Telephone No. +46 8 782 25 00

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

26/02/94

International application No.  
PCT/DK 93/00441

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO-A1-	9311254	10/06/93	NONE		
EP-A1-	0407225	09/01/91	JP-T-	4500608	06/02/92
			WO-A-	9100910	24/01/91
WO-A2-	9100353	10/01/91	AU-B-	638263	24/06/93
			AU-A-	5953890	17/01/91
			EP-A-	0410498	30/01/91